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The role of endogenous H₂S production during hibernation and forced hypothermia

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Reduction of body temperature governs neutrophil retention in hibernating and non-hibernating animals by margination.

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ABSTRACT

Hibernation consists of periods of low metabolism called torpor that are interspersed by euthermic arousal periods. During both deep and daily (shallow) torpor, the number of circulating leukocytes decreases, while circulating cells are restored to normal numbers upon arousal. Here we show that neutropenia during torpor is due solely to lowering of body temperature, since a reduction of circulating also occurred following forced hypothermia in summer euthermic hamsters and rats that do not hibernate. Splenectomy had no effect on reduction in circulating neutrophils during torpor. Margination of neutrophils to vessel walls appears to be the mechanism responsible for reduced numbers of neutrophils in hypothermic animals because the effect is inhibited by pretreatment with dexamethasone. In conclusion, low body temperature in species that natural use torpor or in non-hibernating species under forced hypothermia leads to a decrease of circulating neutrophils due to margination. These findings may be of clinical relevance as they could explain, at in least part, the benefits and drawbacks of therapeutic hypothermia as employed in trauma patients and during major surgery.

INTRODUCTION

Hibernation is an energy-conserving mechanism that promotes survival of endothermic animals during periods of scarce food supply. The hibernation season is characterized by periods of reduced metabolism and body temperature known as torpor bouts, which are interspersed by short (<24 h) arousal periods to normal (euthermic) body temperatures. During bouts of deep torpor, which last for several days to a month, body temperature can fall as low as $\sim -3^{\circ}\text{C}$, depending on species and ambient temperature (1-5). A second form of metabolic depression is “daily torpor”, in which minimum body temperatures typically reach only $\sim 18-30^{\circ}\text{C}$ and torpor bout duration does not exceed 24 h (6). In addition to a suite of physiological changes that occur during torpor (e.g., depressed heart and ventilation rates (7)), immune function is significantly reduced (8). Both the innate and the adaptive arms of the immune system are affected, of which the underlying mechanisms remain to be unraveled. At least part of the reduced immune function *in vivo* can be explained by a substantial drop in numbers of circulating leukocytes (leucopenia) during torpor as demonstrated for all species of hibernating animals studied thus far (7;9-14). Previously, we demonstrated that low body temperature affects the number of circulating lymphocytes during torpor through a reduced plasma level of sphingosine-1-phosphate (S1P), resulting in impaired lymphocyte egress from lymphoid tissues (10).

The explanation for the decrease in number of circulating neutrophils during torpor is less clear. One mechanism could be retention of neutrophils in lungs, because numbers of neutrophils in lungs increase during torpor in hibernating hedgehogs (*Erinaceus europaeus* L.) (15). Studies with mongrel dogs (*Canis familiaris*) also revealed that hypothermia induced a reversible leucopenia due to adherence of cells to the endothelium of mesenteric venules (16). However, whether this mechanism leads to a reduced number of circulating neutrophils during torpor is not known. Therefore, in the present study we determined whether transient neutropenia during torpor (and hypothermia) is due to temporary attachment to vessel walls, or whether alternative mechanisms are involved. We performed experiments in naturally hibernating and in non-hibernating (forced hypothermic) animals to better identify the underlying mechanisms that lead to transient neutropenia during torpor.

MATERIALS AND METHODS

Induction of deep torpor in Syrian hamsters

Male and female Syrian hamsters (*Mesocricetus auratus*) were bred in the animal facilities of the University of Groningen. Animals were housed at an ambient temperature of 21 ± 1 °C and summer photoperiod (L:D-cycle 14:10 h). Torpor was induced by first shortening the L:D-cycle to 8:16 h for ~10 weeks followed by housing at continuous dim light (<5 Lux) at an ambient temperature of 5°C; animals at this stage that did not show signs of torpor behavior were called “winter euthermic”. During hibernation, Syrian hamsters exhibit deep torpor bouts of 3-6 days alternating with arousal periods of approximately 23 hours (17). Torpor-arousal patterns were monitored by movement detectors connected to a computer. Hamsters were sacrificed during winter euthermia, torpor or during interbout arousal (referred to as “arousal”). Experiments with Syrian hamsters were approved by the Institutional Animal Care and Use Committee of the University Medical Center Groningen.

Induction of “daily torpor” in Djungarian hamsters

Male and female Djungarian hamsters were bred in the animal facilities of the Rowett Institute for Nutrition and Health at the University of Aberdeen. All animal work was licensed under the Animals (Scientific Procedures) Act of 1986. Prior to the experiments animals were housed at an ambient temperature of 21 ± 1 °C and a summer-like photoperiod (LD 16:8). “Daily torpor” was induced by shortening the LD cycle to LD8:16 for ~14 weeks at an ambient temperature of 21°C. Visual inspection of torpid behavior (absence of activity and typical torpor posture) was carried out in the middle of the light phase, which is the usual torpor time for Djungarian hamsters. Animals were sacrificed during winter euthermia (time-matched euthermic control for torpid animals), torpor or arousal (± 12 hours following a bout of “daily torpor”).

Rats

Male Wistar rats (*Rattus norvegicus*) weighing 350 – 400 grams were obtained from Harlan Netherlands B.V. Experiments in rats were approved by the Institutional Animal Care and Use Committee of the University Medical Center Groningen.

Forced hypothermia

Summer euthermic Syrian hamsters housed at a LD-cycle of 14:10 h and Wistar rats housed at a L:D-cycle of 12:12 h were anesthetized by intraperitoneal injection of 200 mg/kg ketamine and 2 mg/kg diazepam intraperitoneally. After induction of anesthesia, a catheter was inserted into the jugular vein for blood sampling. Rectal temperature and heart rate (ECG) were monitored continuously (Cardiicap S/5, Datex Ohmeda, USA). Following cannulation, an initial blood draw and installation of the monitoring devices, animals were cooled by

applying ice-cold water on the fur until they reached a body temperature of $\sim 10\text{--}15^\circ\text{C}$. These animals could be rewarmed without ventilatory support. Rats had to be ventilated throughout the procedure to allow safe cooling to a body temperature of $\sim 15^\circ\text{C}$ and subsequent rewarming. Therefore, following intubation, rats were ventilated mechanically (Amsterdam Infant Ventilator; HoekLoos). The tidal volume was set to achieve normocapnia, as verified by capnography and arterial blood gas analysis, with O_2/air (1:2) at a ventilation rate of 50 min^{-1} (0.5 s inspiration time). After reaching the desired low body temperature, animals were immediately rewarmed using a water-based heating mattress. Cooling and rewarming rates were $\sim 1^\circ\text{C}$ per 3 minutes. Blood samples were taken at several body temperatures during cooling, when animals reached the desired low body temperature, during rewarming and at least ten minutes after a euthermic temperature ($>35^\circ\text{C}$) was reached. In another experiment, summer euthermic Syrian hamsters were pretreated 4 hours preceding forced hypothermia with 10 mg/kg dexamethasone or saline intraperitoneally and a baseline blood sample was drawn by orbital puncture. They were then cannulated, cooled and rewarmed as described above, and blood samples were drawn at the induction of anesthesia, upon reaching the desired low body temperature and following rewarming. In all experiments, blood ($\sim 200\text{ }\mu\text{l}$) was collected via the jugular vein catheter in EDTA-coated cups (minicollect EDTA-K3; Greiner Bio-One, Alphen a/d Rijn, The Netherlands) for automated hematological analysis using a Sysmex XE-2100 (Sysmex, Etten-Leur, The Netherlands) (18;19).

Splenectomy

Splenectomies were performed on summer euthermic and torpid Syrian hamsters. After induction of anesthesia with isoflurane (2-2.5 % in O_2), flunixin-meglumin was given subcutaneously (4 mg/kg) for analgesia. A small abdominal midline incision was made, the arteries and veins towards and from the spleen were ligated and the spleen was resected followed by wound closure. Splenectomized summer euthermic animals were allowed to recover at $\sim 20^\circ\text{C}$ (LD-cycle 14 h:10 h) for at least a week before induction of torpor. They were sacrificed during their third torpor bout, which was 60.3 ± 8.1 days following splenectomy. Torpid animals were splenectomized during their third torpor bout. These animals were kept $<10^\circ\text{C}$ using ice-packs. Animals recovered in a climate-controlled room at an ambient temperature of $\sim 5^\circ\text{C}$ (continuous dim light) and were sacrificed directly after reaching euthermia ($>33^\circ\text{C}$) following arousal. The perioperative handling of animals induced the onset of arousal, which started immediately followed weaning from anesthesia.

Sacrificion and sample collection

Syrian hamsters were sacrificed by intraperitoneal injection of an overdose of pentobarbital followed by decapitation. Djungarian hamsters were sacrificed with CO_2 and then decapitated. Rats were sacrificed after the last blood sample was drawn by decapitation

while being anesthetized. After euthanasia (but before decapitation), body temperature was measured rectally and blood (~200 µl) was collected via cardiac puncture in EDTA-coated cups (minicollect EDTA-K3). This blood sample was analyzed with an automated hematology analyzer (Sysmex XE-2100) (18;19). Differential leukocyte counts were validated manually using Wright-Giemsa stained blood smears. Spleens from torpid and aroused Syrian hamsters were removed, snap-frozen in liquid nitrogen and stored at -80°C.

Statistical analysis and data presentation

Data are presented as mean \pm standard error of the mean (SEM). Group comparisons were performed using a two-tailed independent samples Student's T-test in the case of two groups. In the case of more than two groups, a One-Way ANOVA with post-hoc Tukey on normally distributed variables (based on Homogeneity of Variances) or a Kruskal-Wallis test followed by a Mann-Whitney U test in the case of non-normally distributed variables was performed. Statistical differences were calculated using SPSS 20.0, where $p < 0.05$ was considered significantly different.

RESULTS

Deep torpor and “daily torpor” are associated with a reduced number of circulating leukocytes

Body temperatures of Syrian hamsters during winter euthermia and deep torpor were $34.0 \pm 0.5^\circ\text{C}$ ($n = 8$) and $6.2 \pm 0.1^\circ\text{C}$ ($n = 8$), respectively ($p < 0.01$). Numbers of circulating leukocytes were 2.92 ± 0.71 ($\times 10^6/\text{ml}$) during winter euthermia and 0.12 ± 0.04 ($\times 10^6/\text{ml}$) in deep torpor ($p < 0.01$). After 5-6 days of torpor, animals aroused spontaneously and the number of leukocytes increased rapidly to 2.72 ± 0.34 ($\times 10^6/\text{ml}$; $n = 8$) ($p < 0.01$), i.e. to the same level as seen in winter euthermic animals. The numbers of erythrocytes in the blood were similar in winter euthermic hamsters ($8.96 \pm 0.42 \times 10^9/\text{ml}$), torpid hamsters ($9.05 \pm 0.26 \times 10^9/\text{ml}$) and in aroused animals ($7.71 \pm 0.49 \times 10^9/\text{ml}$).

Body temperatures of Djungarian hamsters during winter euthermia and “daily torpor” were $35.0 \pm 0.2^\circ\text{C}$ ($n = 6$) and $25.2 \pm 1.3^\circ\text{C}$ ($n = 8$), respectively ($p < 0.01$). The number of circulating leukocytes declined from 9.50 ± 0.85 ($\times 10^6/\text{ml}$) in winter euthermia to 5.02 ± 0.41 ($\times 10^6/\text{ml}$) in torpor ($p < 0.01$), and then rose again to winter euthermic levels 8.22 ± 0.96 ($\times 10^6/\text{ml}$) upon arousal ($n = 5$) ($p < 0.01$). Erythrocyte counts in Djungarian hamsters remained stable during hibernation, being 9.45 ± 0.33 ($\times 10^9/\text{ml}$) in euthermia, 8.80 ± 0.31 ($\times 10^9/\text{ml}$) during “daily torpor” and 9.67 ± 0.15 ($\times 10^9/\text{ml}$) upon arousal. These results suggest that leucopenia is not restricted to deep torpor, but also occurs in animals exhibiting “daily torpor”.

Deep and “daily torpor” are associated with neutropenia

Leucopenia during torpor is due to a decrease in numbers of all major classes of leukocytes, including neutrophils, lymphocytes and monocytes (7;9-14). In Syrian hamsters the number of circulating neutrophils decreases during entry into deep torpor as body temperature falls, and numbers are correlated strongly with the body temperature ($P = 0.679$; $n = 79$; $p < 0.01$). The number of circulating neutrophils is 0.92 ± 0.31 ($\times 10^6/\text{ml}$; Figure 1A) during winter euthermia and 0.02 ± 0.00 ($\times 10^6/\text{ml}$) ($p < 0.05$; Figure 1B) during torpor. Numbers of circulating neutrophils are restored to 1.50 ± 0.35 ($\times 10^6/\text{ml}$) ($p < 0.01$; Figure 1C) upon arousal. Interestingly, arousal from torpor is associated with a greater number of circulating neutrophils compared with animals that had not yet entered torpor (i.e. winter euthermic animals) or following rewarming from forced hypothermia (see below). During “daily torpor” in the Djungarian hamster, the number of circulating neutrophils falls significantly during torpor as compared to winter euthermic animals and is followed by full restoration upon arousal (Figure 1D).

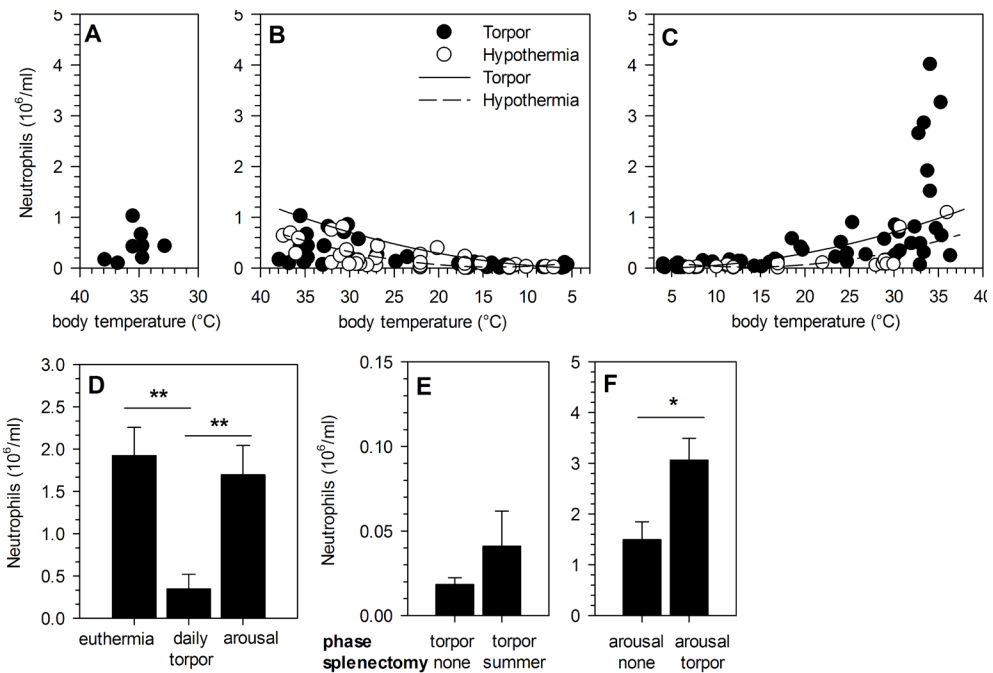


Figure 1. Low body temperature governs a decrease in the number of circulating neutrophils, which is unaffected by splenectomy. Normal number of circulating neutrophils in winter euthermic Syrian hamsters (A); the number of circulating neutrophils decreases gradually during either entrance into deep torpor or forced hypothermia in nonhibernating Syrian hamsters (B); blood neutrophil counts rise upon arousal from torpor and rewarming following forced hypothermia in Syrian hamsters (C); the number of circulating neutrophils is reduced during “daily torpor” followed by restoration upon arousal in the Djungarian hamster (D); splenectomy preceding hibernation, in the summer season, does not affect clearance of circulating neutrophils during torpor (E); splenectomy during torpor leads to an increased number of circulating neutrophils upon arousal in the Syrian hamster (F). Bars represent means \pm SEM of $n = 4-8$ animals per group. * represents significantly different at $p < 0.05$.

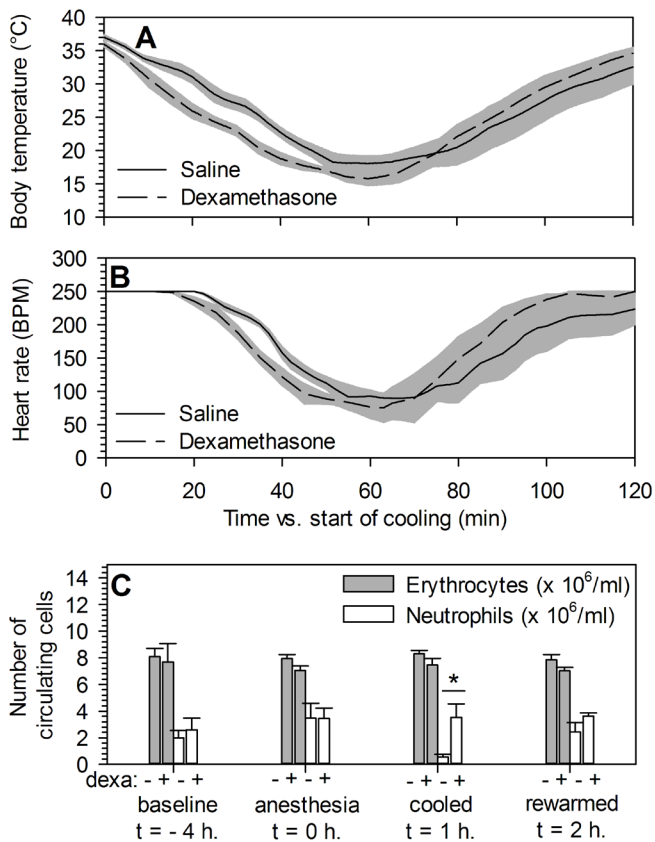


Figure 2. Neutropenia induced by low body temperature in a hibernator is due to margination of neutrophils. Forced hypothermia of anesthetized summer active Syrian hamsters lead to a profound reduction in body temperature that was unaffected by dexamethasone pretreatment intraperitoneally (**A**); lowering of body temperature is associated with a severe drop in heart rate, both in saline as well as in dexamethasone pretreated hamsters (**B**); although hypothermia did not affect erythrocyte counts, the number of neutrophils was transiently reduced by hypothermia, which was precluded by pretreatment with dexamethasone ($p < 0.05$) (**C**). Dexa = dexamethasone (10mg/kg) pretreated animals. Graphs represent means \pm SEM (shown as grey shaded areas or by caps, respectively) of $n = 4$ (saline) and $n = 6$ (dexamethasone) animals per group. * represents significantly different at $p < 0.05$.

Forced hypothermia induces neutropenia in hamsters

The effect of low body temperature on the number of circulating neutrophils was further demonstrated by cooling anesthetized summer euthermic Syrian hamsters ("forced hypothermia"). Cooling to a body temperature of $9.1 \pm 0.8^\circ\text{C}$ induces a decrease in the number of circulating neutrophils as observed in deep torpor: from 0.54 ± 0.09 ($\times 10^6/\text{ml}$; $n = 5$) to 0.02 ± 0.01 ($\times 10^6/\text{ml}$; $n = 5$) ($p < 0.01$; Figures 1B, C). Thus, this finding indicates that simply lowering the body temperature leads to the induction of neutropenia.

The spleen is not the only organ involved in induction or restoration of neutropenia

To evaluate a potential role of spleen in storage of circulating neutrophils during torpor, we examined the effect of splenectomy on the numbers of circulating neutrophils. Syrian hamsters were splenectomized either before entering hibernation or during torpor. Removal of the spleen before hibernation did not affect the number of circulating neutrophils during torpor, i.e. neutrophil numbers in splenectomized torpid hamsters are similar to those in non-splenectomized animals (Figure 1F). In addition, splenectomy during torpor did not prevent the rise in the number of circulating neutrophils upon arousal (Figure 1G). However, following removal of the spleen during torpor, the number of circulating neutrophils was significantly higher in aroused animals that were splenectomized during torpor compared to non-splenectomized animals ($p < 0.05$; Figure 1G). Thus, the spleen is not essential for retention or storage of large numbers of neutrophils during torpor.

The percentage of circulating immature granulocytes is not altered during hibernation

We speculate that if massive apoptosis of neutrophils occurs during torpor, then substantial release of (newly produced) neutrophils from the bone marrow should occur upon arousal to restore the number of circulating cells. To obtain information about the number of newly produced neutrophils, we measured the number of immature granulocytes in the Syrian hamster. Numbers of immature granulocytes are very low in summer, winter euthermic and hibernating (torpid and aroused) animals (detection limit of 0.001×10^6 cells/ml). Although this low number hampers exact quantification of immature granulocytes, it does rule out significant release of immature (neutrophilic) granulocytes from the bone marrow upon arousal. Hence, massive apoptosis of neutrophils seems unlikely to be the sole explanation for neutropenia during torpor.

Low body temperature also drives neutropenia in non-hibernating species (rats)

We found that the number of circulating neutrophils is reduced during deep and “daily torpor” in hibernating hamsters as well as during forced hypothermia in non-hibernating hamsters. To assess whether the induction of neutropenia by low body temperature is a general mechanism not restricted to hibernating species, we induced forced hypothermia in rats (*Rattus norvegicus*), a non-hibernating species. Lowering the body temperature results in a concomitant decrease in the number of circulating leukocytes, which is due to reduced numbers of neutrophils, lymphocytes, monocytes and eosinophils, but not basophils (Table 1). However, the number of circulating erythrocytes remains stable during forced hypothermia. In contrast to hamsters, rats have a higher number of circulating immature granulocytes. Cooling the rats reduced the number of immature granulocytes to values below the detection threshold, although not significantly different from the number prior to cooling (i.e. $< 0.001 \times 10^6$ /ml) (Table 1). Following rewarming, the number of immature granulocytes

Table 1. Forced hypothermia in a non-hibernating animal, the rat, leads to a reduced number of circulating neutrophils, lymphocytes and monocytes. Table shows the body temperature (°C), the number of circulating erythrocytes (x10⁹/ml), leukocytes (x10⁶/ml) and differentiated leukocyte counts (x10⁶/ml) during euthermia (prior to experiment), forced hypothermia and following full rewarming in the Wistar rat. Shown are means ± SEM of *n* = 5 animals per group. Significant differences (*p* < 0.05) between groups are indicated by the printed superscripts (HT = hypothermia, EU = euthermia, REW = rewarming).

	<i>Wistar rat</i>		
	<i>Euthermia</i> (<i>n</i> = 5)	<i>Hypothermia</i> (<i>n</i> = 5)	<i>Rewarming</i> (<i>n</i> = 5)
Body temperature	37.0 ± 0.0 ^{HT}	15.4 ± 0.4 ^{EU,REW}	37.0 ± 0.0 ^{HT}
Erythrocytes	7.81 ± 0.20	6.84 ± 0.72	7.48 ± 0.49
Leukocytes	8.82 ± 1.07 ^{HT}	0.57 ± 0.20 ^{EU,REW}	7.68 ± 1.53 ^{HT}
Neutrophils	1.63 ± 0.49 ^{HT}	0.15 ± 0.04 ^{EU,REW}	2.59 ± 0.75 ^{HT}
Lymphocytes	6.76 ± 0.96 ^{HT}	0.39 ± 0.19 ^{EU,REW}	4.79 ± 1.37 ^{HT}
Monocytes	0.34 ± 0.08 ^{HT}	0.03 ± 0.01 ^{EU}	0.21 ± 0.11
Eosinophils	0.08 ± 0.03	0.00 ± 0.00	0.08 ± 0.03
Basophils	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
Immature Granulocytes	0.012 ± 0.005	0.00 ± 0.00	0.012 ± 0.005

rose to values that were not significantly different from pre-cooling levels (Table 1). The absence of a significant rise in the number of immature granulocytes following hypothermia as compared to baseline values suggests that the restoration of circulating neutrophils upon rewarming is likely not due to release of immature cells from the bone marrow.

Pretreatment with dexamethasone prevents neutropenia during forced hypothermia.

Since apoptosis and reduced release from the bone marrow are unlikely explanations for the kinetics of neutrophils during torpor and arousal, we explored whether temporary retention of cells might lead to neutropenia during torpor. Although the spleen is not involved in the induction of neutropenia during torpor, an alternative explanation for transient neutropenia during torpor and hypothermia is temporary retention of neutrophils in organs due to margination of cells to vessel walls. Thus, we injected summer euthermic Syrian hamsters prior to forced hypothermia with 10mg/kg dexamethasone intraperitoneally to inhibit margination of neutrophils (20-22). As shown in Figures 2A and B, dexamethasone pretreatment did not affect the induction of hypothermia or the associated drop in heart rate. However, it abolished the neutropenia that normally occurs during hypothermia (Figure 2B), but had no effect on the number of circulating erythrocytes (Figure 2C). Since dexamethasone can also stimulate release of neutrophils from the bone marrow in addition to demargination of cells (20-22), we counted the number of immature granulocytes in blood. The number of circulating immature granulocytes was comparable to that during hibernation, around the detection limit of 0.001 x 10⁶ cells/ml. These results support the hypothesis that low body temperature governs neutropenia by margination.

DISCUSSION

In all hibernating animals studied so far, the number of circulating leukocytes decrease after entrance into torpor followed by rapid restoration upon arousal (8). Several studies demonstrated that leucopenia during torpor is due to a reduction in the number of circulating lymphocytes, neutrophils and monocytes (9-12;14;23). Previously, we showed that the drop in lymphocytes during hibernation is driven by low body temperature that directly results in lower sphingosine-1-phosphate (S1P) levels in the blood (10). In the current study, we demonstrate that the reduction in circulating neutrophils is also driven by the decrease in body temperature during torpor, because neutropenia occurs in deep hibernators as well as during daily torpor in the Djungarian hamster. Moreover, neutropenia is also induced by forced hypothermia in summer euthermic Syrian hamsters and in rats, a non-hibernating species.

The mechanism underlying the reduction of neutrophils in blood might be explained by lower production of neutrophils, increased apoptosis and/or temporary retention of cells during torpor. The half-life of neutrophils is estimated to be 14 hours (as measured in mice) (24;25). Therefore, we speculate that if a reduced release from the bone marrow is the sole explanation for neutropenia during torpor, a period of approximately three to five days is required to lead to the low number of circulating neutrophils as observed during torpor. However, the number of circulating leukocytes correlates closely with the reduction in body temperature. Furthermore, ~95% of the leukocytes are cleared from the circulation as soon as the animals approach their torpid body temperature, which is well within 14 hours. Thus, it is unlikely that a lower production rate of neutrophils by the bone marrow contributes significantly to the drop in cell numbers observed during torpor. Neutropenia during torpor is also not explained by massive apoptosis of neutrophils, because there is no difference in the number of immature circulating neutrophils during arousal from deep torpor or rewarming from forced hypothermia in Syrian hamsters or rats, and the fraction of immature granulocytes remains very low. Thus, a reduced production of cells and/or apoptosis cannot explain the rapid induction of leucopenia during torpor or forced hypothermia. Furthermore restoration of circulating neutrophils upon arousal or following rewarming from hypothermia does not appear to be due to rapid regeneration in the bone marrow.

An alternative explanation is that neutrophils are temporarily retained at certain sites during torpid periods and are released again during arousal. Villalobos *et al.* demonstrated a comparable phenomenon to occur in deep hypothermic dogs leading to leucopenia and a reduction in the number of platelets (26). In line with our results, rewarming of the dogs induced restoration of the number of circulating leukocytes and platelets. Comparison of the number of circulating platelets in blood samples derived from aorta, upper and lower vena

cava and capillaries (of the tongue) did not reveal specific retention in the arterial, capillary or venous vascular system. Splenectomizing and hepatectomizing dogs prior to hypothermia reduced the extent of thrombopenia. However, neither splenectomy, nor hepatectomy or splenectomy combined with hepatectomy, precluded thrombopenia induced by hypothermia. Thus, hypothermia leads to reversible clearance of circulating leukocytes and platelets, which does not seem to be confined to arteries, capillaries or veins and in which the spleen and liver do not play a key role.

Our findings also indicate that the spleen does not play a significant role in storage neutrophils during torpor. Splenectomy preceding the hibernation season does not affect the reduction in the number of circulating neutrophils, nor did splenectomy of torpid animals prevent restoration of normal numbers of circulating neutrophils during subsequent arousal to euthermia. Although these results suggest that the spleen does not play an essential role, they do not completely rule out a role in the induction or restoration of neutropenia during hibernation. In contrast, our data support the hypothesis that neutrophils stop circulating during torpor due to reversible adherence to endothelial cells of blood vessel walls (margination), because neutropenia induced by low body temperature was fully blocked by dexamethasone pretreatment in our model of forced hypothermia. Margination depends on the interaction between leukocytes and endothelial cells, due to expression of integrins, selectins and adhesion molecules and detaching forces, such as shear forces due to blood flow velocity (27;28). Studies in rabbits show that under normal circumstances, a substantial number of neutrophils are marginated: the marginated fraction can be as high as ~61% of the number of circulating neutrophils (20). Dexamethasone reduces margination of neutrophils through mechanisms that are not fully understood yet (20-22). Possibly, reduced expression of L-selectin and CD18 on neutrophils (29;30) and endothelial-leukocyte adhesion molecule-1 (ELAM-1) and intracellular adhesion molecule-1 (ICAM-1) on endothelial cells (31), observed after dexamethasone treatment of cells, plays a role in demargination of neutrophils. In addition, glucocorticoids can increase the half-life of neutrophils and stimulate release from the bone marrow through G-CSF (21;22). The number of circulating immature granulocytes remains low after injection of dexamethasone, which suggests that the effect of dexamethasone on hypothermia-induced neutropenia is not due to stimulation of bone marrow release. Thus, low body temperature governs neutropenia by margination, which likely explains the neutropenia associated with torpor.

The mechanism by which low body temperature induces margination is not yet fully understood. In general, margination of neutrophils is promoted by adhering forces such as the expression of adhesion molecules and integrins and is reduced by detaching forces such as increased shear stress by blood flow. Direct effects of lowered body temperature are unlikely to cause neutropenia by means of margination since low temperature reduces

the expression of ICAM-1 in lung *in vivo* (32) and E-selectin on endothelial cells *in vitro* (33;34). However, these effects might be compensated for by indirect effects of low body temperature *in vivo*, such as a reduced cardiac output and the subsequent drop in blood flow velocity that might increase leukocyte-endothelium adhesion. Our data support the hypothesis that lowered body temperature induces margination, an effect which is not restricted to hibernating species, as we also observed this phenomenon in the rat. However, the existence of additional effects, specific for hibernators, cannot be excluded. Yasuma *et al.*, (35) showed that incubation of rat endothelial cells at 37°C *in vitro* with plasma from hibernating, but not from euthermic thirteen-lined ground squirrels leads to upregulation of ICAM-1 expression and increased monocyte adhesion to these cells. These findings suggest that in addition to lowered body temperature, temperature-independent effects of torpor might affect the expression of adhesion molecules, which could potentially promote margination of neutrophils during torpor *in vivo*. In summary, adherence of neutrophils to vessel walls increases when body temperature falls. This finding may explain the reduced number of circulating cells during deep torpor, “daily torpor” and forced hypothermia.

Neutropenia during torpor may also contribute to the diminished function of the innate immune system during torpor. A reduced innate immune function has been demonstrated by intraperitoneal injection of lipopolysaccharide (LPS) during deep torpor, which does not lead to a febrile response as long as the animals remain torpid. However, injection of LPS during arousal induced a febrile response and prolonged the duration of arousal (36). Possibly, reversible reduction of the innate immune system by margination of cells allows conservation of energy while maintaining the potential of rapid reactivation in case of infection. Understanding the mechanisms involved in the reduced function of the immune system may be relevant to the pathophysiological consequences of therapeutic hypothermia (37). Although hypothermia is used clinically to stabilize trauma patients and during major surgery to suppress metabolic activity and limit organ injury during periods of low oxygen supply, it is associated with the occurrence of serious side effects such as fatal thrombosis (38-40), neurological deficits (41), renal injury (42) and increased blood loss (43). Organ injury might be exaggerated by inflammation with influx of neutrophils, in which adhesion (margination) of neutrophils is an important initial step. Indeed, experimental blockade of margination reduces the extent of pulmonary injury following normothermic cardiopulmonary bypass in a sheep model (44). Interestingly, hypothermia in humans leads to the induction of a reversible leucopenia, comparable to hibernating animals and hypothermic rats (45). Possibly, the increased resistance to ischemia/reperfusion and hypothermia of hibernating animals (46-53) prevents the occurrence of cellular injury and the subsequent onset of an inflammatory response, as might occur in humans during treatment with cardiopulmonary bypass (42;51;54-56). Thus, margination of neutrophils induced by low body temperature might be involved in the etiology of organ injury following hypothermia in humans.

CONCLUSION

Low body temperature induces a decrease in the number of circulating neutrophils, which is not restricted to hibernating species. Our data support the hypothesis that neutropenia due to low body temperature is caused by reversible margination of cells, rather than being caused by apoptosis of cells, a reduced release from the bone marrow or retention in the spleen. Despite the fact that hibernators regularly cycle through periods with an extremely reduced body temperature followed by rapid rewarming, no gross signs of organ injury are evident. Translating the underlying mechanisms that govern increased resistance to hypothermic injury may therefore be of major clinical relevance.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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